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Abstract: As research into various aging-associated neurodegenerative disorders reveals their immense pathophysiological complexity, the focus is currently shifting from studying changes in an advanced disease state to investigations involving pre-symptomatic periods, possible aberrations in early life, and even abnormalities in brain development. Recent studies on the etiology of schizophrenia and autism spectrum disorders revealed a profound impact of neurodevelopmental disturbances on disease predisposition, onset and progression. Here, we discuss how a prenatal immune challenge can affect the developing brain-with a selective focus on the impact on microglia, the brain's immune cells-and the implications for brain aging and its associated risk of developing Alzheimer's disease.

DOI: https://doi.org/10.1007/s11481-012-9374-z

BRIEF REVIEW

Impact of prenatal immune system disturbances on brain development

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Keywords: Polyriboinosinic-polyribocytidilic acid, PolyI:C, late gestation, infection, neuronal health, brain aging

Manuscript:	**23 pages**
Abstract:	**106 words**
Total words:	**5767**
Figures:	**2**
References:	**70**

ABSTRACT

As research into various aging-associated neurodegenerative disorders reveals their immense pathophysiological complexity, the focus is currently shifting from studying changes in an advanced disease state, to investigations involving pre-symptomatic periods, to possible aberrations in early life and even abnormalities in brain development. Recent studies on the etiology of schizophrenia and autism spectrum disorders revealed a profound impact of neurodevelopmental disturbances on disease predisposition, onset and progression. Here, we discuss how a prenatal immune challenge can affect the developing brain – with a selective focus on the impact on microglia, the brain's immune cells – and the implications for brain aging and its associated risk of developing Alzheimer's disease.

Microglia in development

The immune system has developed to ensure cells of the innate immune system reside in all organs of the body. The brain, far from being a completely immune-privileged site, as thought earlier, possesses its own immune cells (Ransohoff and Perry, 2009). These are the microglia, and are the only cells in the central nervous system (CNS) that do not originate from the neuroectoderm (Allen and Barres, 2009), but belong to the myeloid lineage. After some debate how these cells populate the CNS– either via entering the developing brain from peripheral circulation as monocytes followed by differentiation (Perry et al., 1985), or multiplying from intraparenchymal precursors that existed before the development of the vasculature (Alliot et al., 1999), recent studies provided supporting evidence that microglia derive from macrophages that originate from the yolk-sac and then colonize the brain (Herbomel et al., 2001; Ginhoux et al., 2010). Still, what percentage of microglia in the adult pool are remnants from these developmental precursors is unknown. While the predominant

view is that microglia are long-lived cells that are not often replenished from the periphery, different studies have shown that, under certain conditions, peripheral bone-marrow derived mononuclear phagocytes (BMDPs) can integrate into the CNS (Priller et al., 2001; Massengale et al., 2005; Prinz and Mildner, 2011). Moreover, differential expression of certain markers, for instance, *Hoxb8*, seem to indicate that at least two different microglial populations exist in the adult mouse brain (Chen et al., 2011), which may have different implications for their function.

Once the microglia colonize the brain, several studies (Pont-Lezica et al., 2011) have shown that contrary to being randomly and evenly distributed throughout the brain, these cells appear to concentrate specifically at certain locations, either being associated with apoptotic neurons and radial glial cells, for example, or being excluded from others, like the lens and photoreceptor layer of the retina. The distribution of microglial cells in the brain show region-specific differences with highest density in the hippocampus, basal ganglia, olfactory telencephelon and substantia nigra, followed by cerebral cortex, thalamus and hypothalamus, and lowest density in cerebellum brain stem areas, and myelinated fibre tracts (Lawson et al., 1990; Lawson et al., 1993). Moreover, there even seems to be a variation in where the majority of the morphologically distinct microglia generally reside. Those with the shortest processes are found in the circumventricular regions, while the most ramified microglia are found in the grey matter (Lawson et al., 1990). This region-specific density, which varies around five-fold, as well as the differences in morphology, appears to be closely linked to the vast number of functions performed by microglia.

During development, they play significant roles in the formation of a healthy working environment in the CNS, and accomplish this in various ways. First, microglia are found in close association with cells undergoing developmental cell death, and have been shown to be capable of phagocytosing apoptotic neurons (Peri and Nusslein-Volhard, 2008). They have

also been shown to control which cells undergo apoptosis, via NGF signaling or triggering oxidative stress (Frade and Barde, 1998; Wakselman et al., 2008). Large microglial populations have also been discovered free of apoptotic cells, and these are thought to have other functions. Those in close association with the developing vasculature have been suggested to play a key role in angiogenesis, as evidenced by the underdeveloped vascular system in mice lacking microglia (Rymo et al., 2011). Others are associated with developing axon tracts throughout the brain (Herbomel et al., 2001), at the right time to influence neurite development and axon remodeling, as well as synaptic pruning in development (Paolicelli et al., 2011). Microglia have been shown to contain large inclusions that often resemble axon terminals and even spines (Tremblay, 2012), and could contribute to experience-dependent remodeling of synapses and developing circuits. In line, members of the complement system, often derived from microglia, have also been shown to be involved in synapse elimination (Stevens et al., 2007). Recent research also showed that mice lacking CX3CR1 (a microglia-specific chemokine receptor) show significant impairments in synaptic development as compared to wild-type littermates (for recent review, Ransohoff and Perry, 2009).

Microglia functions in the adult brain

The close and tightly regulated interaction between microglia and neurons during brain development is maintained in the adult brain. This has been highlighted by several studies recently summarized by Trembay and colleagues (2011), indicating that microglia are actively involved in the remodeling of the perisynaptic environment and fine-tuning of neuronal activity, likely also involving feedback signals provided by the neurons to modulate the state of activity in microglia (Tremblay et al., 2011). This bidirectional communication is achieved through interactions between cell-surface receptors and cell-surface-bound ligands (i.e.

CD200-CD200 receptors), or at a distance after the generation of diffusible ligands, as well as via neurotransmission-associated inhibition involving dopamine and noradrenaline and their respective receptors (for recent review, Ransohoff and Cardona, 2010). In turn, through the release of a multitude of soluble factors including neurotrophic factors chemokines, cytokines, microglia not only perform classical glia functions, but also accomplish a crucial immune function by surveillance of the brain for damage infection, and engulfing infectious agents, dead cells and debris (Streit, 2006, 2009).

Interestingly, the morphology of the cells seems closely related to function. At least two distinct configurations have been observed – highly branched with long, fine processes, or rounded and amoeboid. The former were considered to be "resting microglia" that occur in a healthy, undisturbed CNS. This term later proved to be a misnomer, since these cells were shown to be highly dynamic, forming networks with their processes, while their soma remain more or less stationary, that probe and test the entire brain in a matter of a few hours (Nimmerjahn et al., 2005; Schlegelmilch et al., 2011; Wirenfeldt et al., 2011). The onus is to designate these microglia more appropriately as "surveying microglia" (Hanisch and Kettenmann, 2007). The amoeboid cells are indeed more active in the immune sense, with the surveying glia transforming to this phenotype, possessing upregulated antigen presenting molecules, when phagocytosis is required. This rapid conversion to the amoeboid form is termed "activation of microglia" and can be triggered by a variety of stimuli, ranging from infection and trauma to ischemia or any disturbance in the normal functioning of the brain (Kettenmann et al., 2011). The abrupt response to neuronal injury could also be related to a disrupted cell-cell contact and the concomitant loss of neuron-mediated inhibition of microglia (for recent review, Ransohoff and Cardona, 2010). Whether these properties also relate to the observed differences in the basal activation state of microglia in white versus grey matter (Carson et al., 2007), remains to be determined. However, it is conceivable that

these functional differences are linked to the expression profile of cell surface markers, through which microglia are not only able to control distinct population of neurons but also modulate a variety of cellular and immune functions (for recent review, Lynch, 2009). For instance, microglia can promote the infiltration of circulating cells into the CNS (upregulation of CD11b), can be non-phagocytic and produce proinflammatory molecules (CD40), or be phagocytic and motile (MHCII), as shown for the highly effective clearance of In the adult brain, microglia are responsible for all apoptotic debris, including the majority of new born neurons (Sierra et al., 2010).

Based on morphology, it is thus possible to differentiate microglia into functionally different states including amoeboid (surveilling) and ramified (activated) forms. A third potential state has recently been hypothesized, by postulating that microglia can devolve from their "surveying" state into an "alerted" one, before becoming fully activated. This resembles the process of priming of macrophages in the periphery, where activation occurs in two steps – priming, followed by triggering (Dilger and Johnson, 2008). A "primed" microglial cell would thus react to a greater degree than a non-primed one. In line with these hypothesis, once activated, microglia tend not to return all the way to their "surveying" state, thereby eliciting elevated responses to the next stimulus (Schwartz et al., 2006). Microglia differ, however, from peripheral immune cells in their preference for humoral responses rather than eliciting the classical cytotoxic responses, which involves the ingestion of antigens, exiting the tissue and entering the draining lymph nodes, where stimulation of naive T cells occurs. This happens perforce due to the nature of the CNS environment – where the neurons cannot regenerate as easily as cells in the periphery, and collateral damage cannot be repaired.

Microglia in Disease and Aging

Several diseases, not only those of the CNS, appear to impact on microglial activity and function. Infectious diseases of the CNS (Mariani and Kielian, 2009), conditions of neuropathic pain (Ji and Suter, 2007), even air pollution (Block and Calderon-Garciduenas, 2009) can create environments where microglia become highly activated, and after a point, cannot protect the CNS any more. Looking at two neurodegenerative diseases – MS and traumatic spinal cord injury (SCI) – the role of microglia in repairing the damage is highlighted. In cases of MS or other demyelinating conditions, it has been shown that there exists a subset of microglia at the site of demyelination that can recruit oligodendrocyte precursor cells and phagocytose damaged neurons (Olah et al., 2011). In SCI, cells of the myeloid origin have been shown to play a role in intraspinal trafficking past the injury (Hawthorne and Popovich, 2011).

Beyond diseases, aging also represents a critical "primer" of microglia activity, and is also accompanied by microglial dystrophy and degeneration, a feature that appears to be accompanied by diminution of microglial neuroprotective functions and hypothesized to significantly contribute to neurodegenerative changes characteristic of AD (Streit, 2004). A combination of microglia senescence on one side and priming-related hyperactivity on the other side is therefore expected to have a detrimental influence on neuronal health. This represents a highly relevant theory regarding AD pathogenesis because it integrates aging as the most important risk factor of this progressive neurodegenerative disease. However, the molecular mechanisms underlying aging-associated microglia dysfunction are largely unknown. Besides a decline in proteasome activity and other aging-associated impairments in protein homeostasis (Stolzing and Grune, 2003), limited information is available regarding the lifespan of microglia (Streit, 2006). It has been shown that nerve injury induces – beside recruitment of bone marrow-derived precursors that can infiltrate the brain - a burst in mitotic

activity and a wave of proliferation that is followed by apoptotic cell death of microglia (Graeber et al., 1988; Gehrmann and Banati, 1995; Lassmann et al., 1995). This represents an essential defense and wound healing mechanism shown to be maintained in aged rodents (Conde and Streit, 2006) and suggested to be required to keep the number of these endogenous immune defenders of the CNS constant. It is conceivable, therefore, that repetitive exposures to brain injury and inflammation induces replicative senescence, eventual loss of mitotic activity and degeneration of microglia during aging. Evidence for this hypothesis has been provided by *in vitro* findings showing that microglia stimulation by mitogens induces telomere shortening (Flanary and Streit, 2004). Recent data also showed that interferon-γ exposure is sufficient to trigger activation-induced microglia cell death (Yun et al., 2011), in line with postmortem investigations of AD patients showing caspase activity in plaque-associated microglia in AD (Yang et al., 1998).

Specifically with respect to AD, the significance between the association of microglia with amyloid plaques has long been debated. Several studies have looked at the possibility of using the phagocytic ability of microglia therapeutically to clear the plaques, but largely, this doesn't seem to have the desired results on cognition. It has further been observed that plaque-associated microglia still possess their processes at the interface, and are capable of phagocytosing Aβ peptides (Bolmont et al., 2008). However, it has also been demonstrated a that microglia show an age-dependent reduction in their ability to clear Aβ peptides, despite preservation of functional phagocytic activity and even adherence to Aβ plaques (Floden and Combs, 2011), underscoring the importance of aging for microglial health and activity.

Prenatal PolyI:C model of accelerated aging

Interestingly, degeneration of microglia has also been reported in neurological disorders without major neurodegenerative changes, such as schizophrenia, (Wierzba-Bobrowicz et al., 2004), or associations with aging. Besides genetic risk factors, maternal infection during pregnancy represents one of the currently most extensively studied risk factor in experimental schizophrenia research. This is based on a large body of epidemiological data that has provided compelling evidence for enhanced risk of schizophrenia following prenatal exposure to infection with various viral or bacterial pathogens, or genital and/or reproductive infections (Brown et al., 2006; Patterson, 2007; Boksa, 2008). This data indicates that the early inflammatory insult not only strongly affects the developing nervous system and predisposes the offspring to psychosis-related behavioural abnormalities in adulthood, but likely also critically influences the proliferation, maturation and function of the developing immune system. The findings of significantly elevated levels of degenerating microglia seen in frontal and temporal cortices of schizophrenia patients compared to healthy subjects (Wierzba-Bobrowicz et al., 2004) indeed supports the hypothesis that early prenatal infection-induced expression of pro-inflammatory cytokines and other mediators of the innate immunity might reduce the proliferative capacity of microglia and hence reduce their lifespan.

To investigate the impact of a prenatal infection on brain and immune system development, we established a mouse model that is based on the critical contribution of the gestational stage during which the maternal immune challenge occurs. It builds on findings reported by other groups that demonstrated a wide spectrum of behavioural, neuroanatomical, and neurochemical changes following exposure to viral or bacterial pathogens in rats and mice that mimicked critical phenotypic symptoms of schizophrenia (for recent review, (Meyer et al., 2009). Based on the highly comparable phenotype induced by the various types of

pathogens we reasoned that the downstream effects, i.e the production of pro-inflammatory cytokines might be the critical common denominator of the behavioural and neurochemical changes described. To test this, we employed a viral mimic, Polyriboinosinic-polyribocytidilic acid (PolyI:C), a double-stranded RNA shown to bind to Toll-like receptor 3 (TLR3), leading to activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and production of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-12 (Alexopoulou et al., 2001; Okun et al., 2011). Given the critical processes during the development of the brain and its resident immune cells, we decided to contrast early, mid- and late-gestational stages. We hypothesize that the developmental stage critically determines the phenotype of the adult offspring, allowing us to test whether prenatal infections may – in addition to the widely accepted role in the etiology of neuropsychiatric diseases – also contribute to brain aging and neurodegenerative diseases.

Our first findings indeed confirmed that the time of maternal immune challenge critically determines the patterns of fetal immunological, juvenile neurochemical, as well as adult behavioural abnormalities displayed by the offspring (Meyer et al., 2006). While the PolyI:C challenge during early-gestation (gestational day 6, GD6) resulted in a high incidence of abortion, a viral-like infection during mid-gestation (GD9) confirmed the precipitation of psychosis-related symptoms in the adult offspring, in line with previous findings by other groups (Borrell et al., 2002; Shi et al., 2003; Zuckerman et al., 2003; Zuckerman and Weiner, 2005; Ozawa et al., 2006; Romero et al., 2007). A late-gestational (GD17) immune challenge resulted in a behavioural phenotype characterized by cognitive impairments, elevation of fetal IL6, IL10, and TNFα, enhanced apoptosis in the dentate gyrus, as well as a decline in Reelin-positive cells in juvenile brains (**Fig. 1**). We showed for the first time that the fetal brain can directly contribute to the specific changes in inflammatory cytokine protein levels after late-gestational but not mid-gestational immune challenge, suggesting a close correlation between

acute immunological modulators and histopathological changes in the juvenile offspring. We also reported that the mid- and late-gestational infections had a differential impact on the developing neurotransmitter systems (Meyer et al., 2008). Using immunohistochemical approaches and unbiased stereological evaluations, we provided first evidence that a prenatal immune challenge around mid-gestation has a stronger impact on the prefrontal dopamine receptor D1 expression as compared to the treatment effect at GD17. These neurochemical alterations were accompanied with behavioural abnormalities with relevance to positive symptom clusters characteristic of schizophrenia. On the other hand, an *in utero* immune challenge at GD17 preferentially affected the expression levels of the hippocampal NR1 subunit of the NMDA receptor as compared to GD9 and control treatments, which were paralleled by impairments in working memory performance. These studies revealed also that a prenatal immune challenge during late gestation resulted in acceleration of aging, as indicated by the earlier formation of Reelin-positive extracellular amyloid-like deposits, a neuropathological feature detected in the hippocampal formation in several species that is accompanied by a reduction in Reelin-expressing neurons (Knuesel et al., 2009; Knuesel, 2010). Both neuropathological alterations significantly correlated with hippocampus-dependent episodic-like memory deficits (Knuesel et al., 2009). We found that healthy and cognitively normal subjects were able to sufficiently clear and degrade these deposits, whereas cognitively impaired aged mice failed to do so. Our results showed for the first time that prenatal exposure to infection during late embryonic developmental stages is not only an important factor in the segregation of certain behavioural symptom clusters with relevance for neuropsychiatric disorders but does also represent a critical driving force of aging-related neuropathological processes.

Effects of PolyI:C *in vitro* and *in vivo* on Microglia

These observations indicated that the effect of PolyI:C on microglia and astrocytes could be highly relevant for such neuropathology. Supporting this view, our studies with primary cultures of these cells revealed that PolyI:C could trigger the morphological changes underlying the transformation of microglia into their activated state, as compared to the ramified morphology of the untreated, or saline treated, surveying microglia, but not changes in astrocyte morphology (**Fig. 2**; Vogel et al., unpublished data). The implication here is that PolyI:C induces a strong and distinct immune response through the TLR3 found on microglia, but saline, a likely stressor, activates different cellular pathways, leading only to a mild or non-selective immune response.

The mRNA of different TLRs has previously been shown to be enriched in the vicinity of amyloid plaques (Frank et al., 2009), likely reflecting their localization on microglia. Our own observations in aged prenatally immune-challenged mice reveal a striking overlap between microglial and TLR3 immunoreactivity (Vogel et al., unpublished data). Besides establishing a chronic adverse neuronal environment, the prenatal immune challenge might prime microglia and thereby alter their ability to react to different stimuli and create an innate memory allowing a faster and exaggerated response upon a second challenge; changes highly comparable to mechanisms in the adaptive immune system. The strong glial response may then have devastating effects on surrounding neurons resulting in demyelination and apoptosis due to release of ROS.

New Perspectives and Future Outlooks

Having seen how delicately balanced the immune system is in the CNS, it becomes more evident that inflammation at critical points during development can have a serious and long-lasting impact on microglial function, and thus, on how the brain ages. Studying microglia during development and understanding the different aspects of their priming and activation could provide insights to disorders that set in much later in life, like AD. Everyone's goal of healthy aging seems thus to depend more on proper developmental processes than so far anticipated.

METHODS

Animals All experimental procedures were approved by the local authorities of the Cantonal Veterinary Office in Zurich and are in agreement with the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985).

Preparation of primary neuronal cultures Pregnant rat dams were sacrificed at gestation GD18. Rat embryos were decapitated, the brain removed and immediately put in ice-cold PBS/glucose. The dissected cortices of 2 to 4 animals were transferred into a tube containing ice-cold PBS/glucose. The PBS/glucose was aspirated and the cortices were passed through a 100 μm and a 70 μm cell strainer in 50 ml DMEM supplemented with 20% FCS, L-Glut and gentamicin (all from Gibco Life technologies, Invitrogen Inc.). The cell suspension was seeded in a 170 cm^2 cell culture flask. After 7 days in culture, the medium was changed to 50 ml astrocyte culture medium. The astrocytes were grown for another 5 to 7 days in vitro before the microglia were mechanically detached using a plate shaker running at 2000 rotations per minute (rpm) for 30 minutes. The medium was aspirated, the attached astrocytes were once washed with pre-warmed PBS and 9 ml trypsin (Sigma-Aldrich) diluted in PBS

added for 5 minutes at 37°C. In order to inactivate the trypsin, 1 ml FCS was added. The astrocytes were collected and spun down for 5 minutes at 1100 rpm. The supernatant was removed and the astrocytes were gently dissociated in astrocyte culture medium with a fire-polished Pasteur pipette. 10 µl of the single cell suspension was mixed with 10 µl Trypan Blue dye exclusion medium and the live cells were counted using a Neubauer chamber. The suspension was diluted to 50'000 cells per coverslip and 200 µl was plated on Poly-L-Lysine (Sigma-Aldrich) coated glass coverslips. After 2 hours in vitro, the coverslips were transferred in astrocyte culture medium in a 12-well plate. The culture plates were maintained at 37°C in an atmosphere of 5% CO_2. After 3 to 7 days, the astrocytes were fixed and stained to assess the purity of the culture.

Cell stimulation PolyI:C (200 µg/ml, P9582, Sigma) was solved in 0.9 % pyrogen-free NaCl (B. Braun, 534534) and directly added to the medium of primary astrocyte cultures after 3 days in vitro.

Immunocytochemistry At 7 days in vitro, the cells were fixed for 15 minutes with 4% PFA at RT and rinsed 3 times with PBS. Subsequently, the cells were permeabilized 4 to 5 minutes with 0.2% Triton X-100 solution (Fluka) in PBS supplemented with 10% Normal Goat Serum (NGS; Serotec) at RT. The cells were then incubated with the primary antibody (rabbit anti-Iba1, 1:4000, Wako Nr. 019-19741; mouse anti-GFAP, 1:5000, Dako, Z334) diluted in PBS containing 10% NGS for 90 minutes at RT. In the following, the cells were washed 3 times 10 minutes with PBS and then incubated with the secondary goat-anti-rabbit-Cy3 (Jackson ImmunoResearch Inc, 1:500) and goat-anti-mouse-Alexa488 (Invitrogen Inc, 1:1000) antibodies diluted in PBS containing 10% NGS for 30 minutes at RT. The cells were dried and covered upside-down with fluorescent mounting medium containing DAPI (Thermo Scientific Pierce Inc.) on glass slides.

Microscopy and ditital imaging Triple-immunofluorescence labelings were visualized by confocal microscopy (LSM-710, Zeiss, Jena, Germany) using a 40x (NA 1.3) and 63x (NA 1.4) and sequential acquisition of separate channels. Z-stacks of consecutive optical sections were summed and projected in the z dimension (maximal intensity) and merged using the image analysis software Imaris (Bitplane, Zurich, Switzerland) for visual display. Cropping of images, adjustments of brightness and contrast were identical for each labeling and done using Adobe Photoshop.

ACKNOWLEDGEMENTS

This study was supported by the Swiss National Science Foundation, Grant Nr 310030-132629 (IK), and National Center for Competence in Research (NCCR project 2, Alzheimer's Disease, IK). The authors thank Prof. Jean-Marc Fritschy for his advice, support and inputs regarding this project. The authors also wish to thank all the people of the Animal Services of the Institute of Pharmacology and Toxicology for animal husbandry and care.

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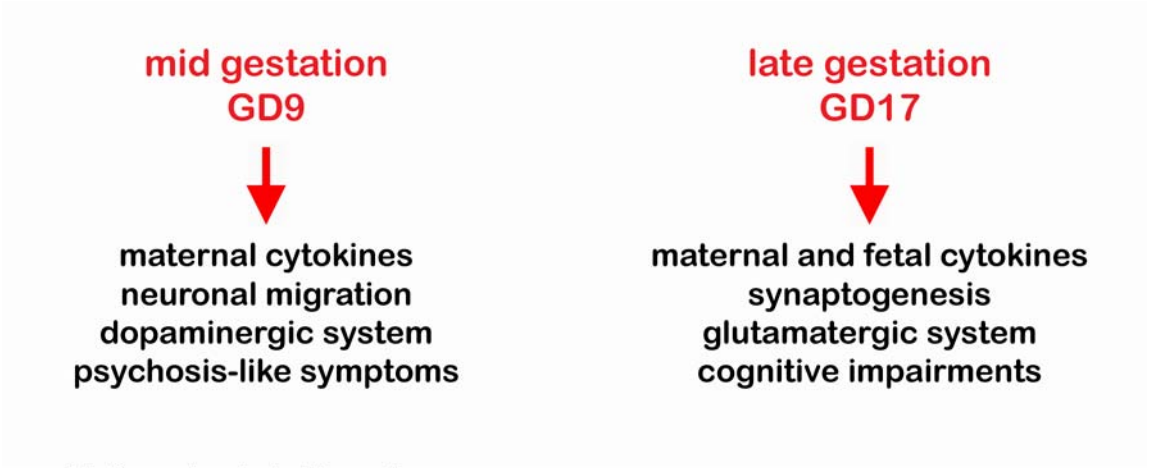
FIGURE LEGENDS

Figure 1: Schematic representation of the critical differences resulting from a prenatal immune challenge at different time periods in gestation.

Figure 2: Glial cells in prenatal astrocyte cultures undergo morphological change upon immune stimulation. **A - B**, Immunofluorescence staining of astrocytes using anti-GFAP antibody (green) and microglia labeled with anti-Iba1 antibody (red) in astrocyte cultures prepared from E18 rats. The nuclei were counterstained with DAPI (blue). Immune challenge with PolyI:C altered the ramified phenotype found in untreated and NaCl-treated cultures into an amoeboid-like appearance indicative of activated microglia. Note, that also GFAP-positive astrocytes change their morphology following PolyI:C exposure. Scale bar: **A**: 50 μm, **B**: high magnification image: 15 μm.



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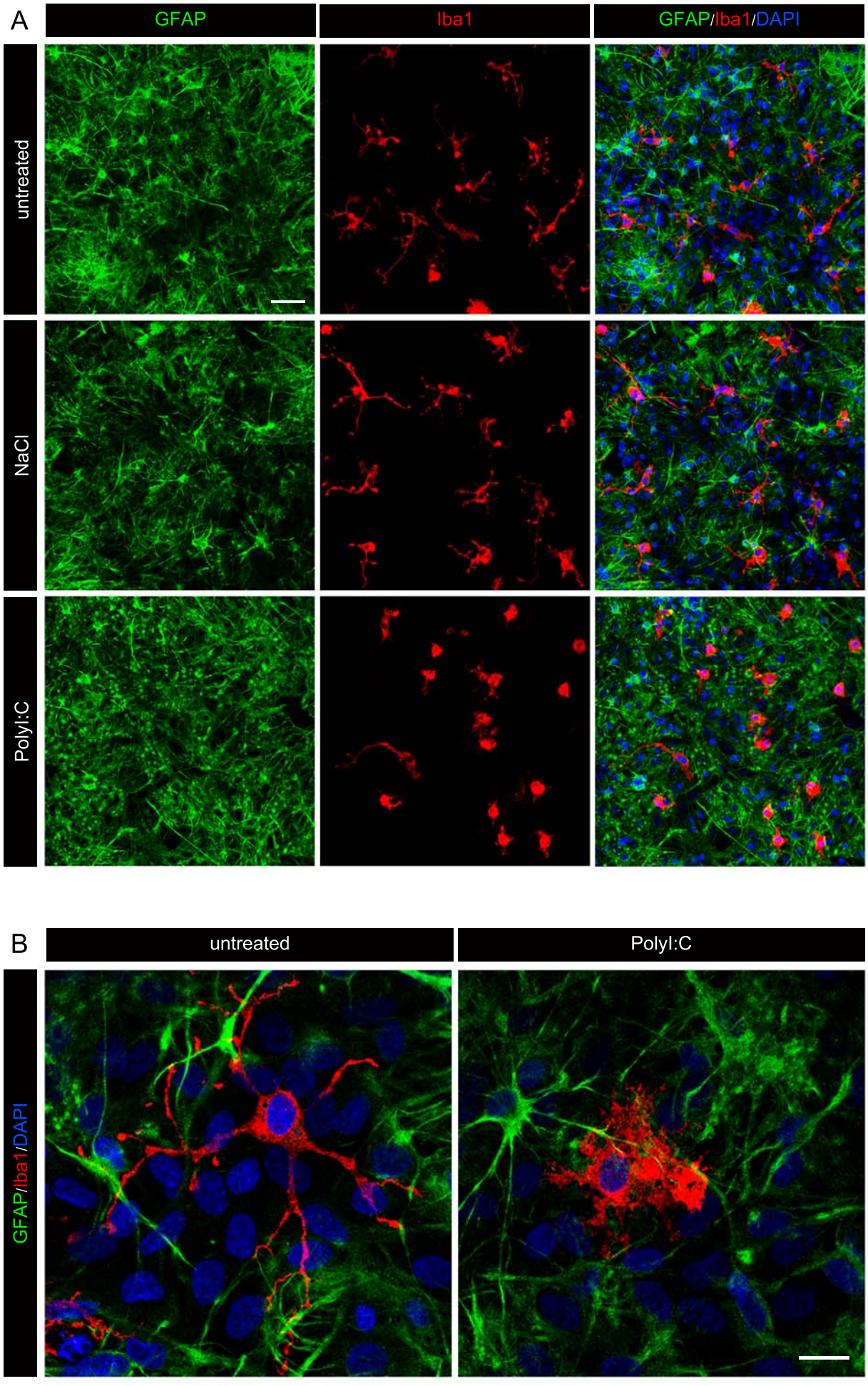

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